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doi: 10.1016/j.abb.2010.03.009

The solubility of nucleobases in aqueous arginine solutions

Atsushi Hirano [a], Hiroko Tokunaga [b], Masao Tokunaga [b], Tsutomu Arakawa [c], Kentaro Shiraki [a,*]

[a] Institute of Applied Physics, University of Tsukuba, Tsukuba, Ibaraki 305-8573, Japan

[b] Laboratory of Applied and Molecular Microbiology, Faculty of Agriculture, Kagoshima University, Kagoshima 890-0065, Japan

[c] Alliance Protein Laboratories, 3957 Corte Cancion, Thousand Oaks, CA 91360, USA.

Corresponding author. Tel.: +81 29 853 5306; fax: +81 29 853 5306.

E-mail address: shiraki@bk.tsukuba.ac.jp (K. Shiraki).

Abstract

Arginine is widely used in refolding, purification, analysis and formulation of proteins, primarily because of its ability to suppress protein–protein and protein–surface interactions. Such a property of arginine is at least partially attributable to its affinity for the aromatic groups present in proteins. Herein, we have extended its study further to characterize arginine in terms of its affinity for heteroaromatic compounds, using nucleobases as a model compound. Arginine increased the solubility of all nucleobases tested when these nucleobases were electrically neutral, indicating that arginine interacts favorably with these heteroaromatics. The observed effects of arginine on the solubility of nucleobases suggest that arginine should stabilize the melted state of nucleic acids, in which the nucleobases are more solvent-exposed. In an acidic solution, to our surprise, arginine decreased the solubility of positively charged nucleobases.

Keywords: Arginine; Drug; Nucleic acid; Nucleobase; Solubility; Transfer free energy.

Introduction

Arginine is widely used in research and development of proteins, such as refolding, chromatography, analysis, and formulation of proteins. Such applications are based on

the ability of arginine to suppress protein–protein and protein–surface interactions [1–8]. Various approaches to address the mechanism of arginine effects have been performed, namely a study of the preferential interaction of arginine with proteins [9] and comparison of chemical structures of arginine derivatives in relation to their effects on protein aggregation [10–12]. The transfer free energy concept, established by a pioneering work of Nozaki and Tanford in the 1960s and 1970s [13–16], revealed that arginine interacts favorably with aromatic groups that are present in proteins, i.e. phenylalanine, tyrosine and tryptophan, through the π–electron of these groups [6].

Recently, we observed that arginine increases the solubility of low molecular weight aromatic compounds [17,18]. The results, which confirmed the affinity of arginine for aromatic groups, further suggested that arginine is applicable to the progress of drug development because the poor solubility of some drugs, namely biopharmaceutical classification system (BCS) type II and IV drugs, exhibit low bioavailability. However, those earlier studies have been limited to aromatic hydrocarbons, while many drug substances are also composed of heteroaromatics. Consequently, we studied the ability of arginine to modulate the solubility of heteroaromatic compounds using nucleobases as a model compound. The observed effects of arginine present important implications, not only in the ability to change the solubility of heteroaromatic drug substances, but also in

the interaction of guanidinium group of arginine with heteroaromatic ring-containing compounds, as discussed in this paper.

Materials and methods

Chemicals

For this study, adenine, guanine, thymine, cytosine, uracil, arginine hydrochloride (HCl), lysine HCl, glutamic acid, aspartic acid, glycine, NaCl, guanidine HCl and trisodium citrate dihydrate were used (Wako Pure Chemical Industries Ltd., Osaka, Japan), along with sodium dihydrogenphosphate dehydrate (Nacalai Tesque Inc., Kyoto, Japan). All compounds were of the highest commercially available grade.

Solubility measurement

The nucleobase solubilities were determined at 25 °C in the presence of 50 mM citrate–phosphate buffer as a function of pH or amino acid concentration. Excess amounts of the nucleobases were suspended in the buffer containing various concentrations of amino acids, NaCl or guanidine HCl and incubated at 40 °C for 1 h to accelerate dissolution. The suspension was brought to the solubility equilibrium at 25 ˚C for 24 h incubation. The suspension was centrifuged at 25 °C and 16,000*g* for 20 min to obtain saturated supernatants. Concentrations of the nucleobases in the supernatants were

determined by absorbance at 260 nm for adenine, 272 nm for guanine, 264 nm for thymine, 266 nm for cytosine, and 259 nm for uracil using a UV-vis spectrophotometer (ND-1000; NanoDrop Technologies, Inc., Wilmington, DE, USA). The solubilities were then calculated from the standard curves determined for each nucleobase.

Calculation of transfer free energy

Transfer free energy ΔG_{tr} of the nucleobases from the buffer solution to the amino acid solutions was calculated according to the following equations.

$$\Delta G_{tr} = \mu_a^0 - \mu_w^0 = -RT\ln(x_a / x_w),$$

$$\begin{cases} \mu_w = \mu_w^0 + RT\ln x_w \\ \mu_a = \mu_a^0 + RT\ln x_a \end{cases},$$

$$\begin{cases} x_w = n_{base,w} / (n_{base,w} + n_{H_2O,w}) \\ x_a = n_{base,a} / (n_{base,a} + n_{H_2O,a} + 2n_{a,a}) \end{cases}.$$

In the equations shown above, μ_i signifies the chemical potential of nucleobase in the presence of *i*, and subscript *w* and *a* respectively denote the buffer solution and various concentrations of the amino acid (here arginine, lysine, glutamic acid, aspartic acid or glycine). Therein, μ^0 and *x* are the standard chemical potential and the mole fraction at saturation. $n_{j,i}$ is the molarity of component *j*, where subscript *base*, H_2O and *a* depict the nucleobase, water, and the amino acid in the presence of *i*. In addition, *R* is the gas constant; *T* is the absolute temperature. For example, μ_w represents the chemical potential

of a nucleobase in a buffer solution and $n_{base,w}$ is the molarity of a nucleobase in the same solution. The activity coefficient was considered to be close to unity because of the poor solubility.

DNA digestion

The deoxyribonuclease (DNase) activity was assayed by measuring the liberation of acid-soluble oligonucleotides from deoxyribonucleic acid (DNA). The reaction mixture (60 μl) contained calf thymus DNA (final concentration of 0.4 mg/ml), 160 mM acetate buffer (pH 5.0), 4 mM ethylenediaminetetraacetic acid (EDTA) and DNase II. After incubation at 37 °C for an appropriate time, 25 μl of the mixture was mixed with a 12% perchloric acid for a final concentration of 2.5%. The mixture was chilled for 15 min and clarified by centrifugation at 4 °C for 10 min at 18,000*g*. A 20 μl of aliquot of the supernatant was diluted with 480 μl of distilled water and the absorbance at 260 nm was measured. Absorbance of the control solution was obtained from the sample solution without the enzyme. The absorbance value of the control was subtracted from that obtained from the respective sample solution. The enzymatic activity was defined as the final calculated absorbance.

Results

The solubilities of nucleobases at neutral pH

Nucleobases have poor aqueous solubilities. Table 1 presents solubilities of adenine, guanine, cytosine, thymine and uracil in citrate–phosphate buffer solution (50 mM, pH 7). All the nucleobases showed low solubilities of less than 100 mM. Among them, guanine was marginally soluble in the buffer solution. The solubility of the nucleobases determined here was compared with the literature values previously reported [19,20] (Table 1). Except for the guanine solubility, the present results were almost identical to the literature values. The observed solubility of guanine was about 3-fold higher than the literature value. In the present study, the nucleobases were first solubilized at 40 °C and brought to equilibrium at 25 °C; hence it is possible that guanine precipitation may still be occurring at the time of solubility measurement. However, it was confirmed that a prolonged incubation had no effect on the solubility of all five nucleobases, including guanine. Why is the guanine solubility 3-fold higher than the literature value? A careful examination showed that the solubility of guanine depended on the amount of powder in the initial solubilization. When a large excess of guanine was used, this higher value was observed. When a slight excess was used, a value close to the literature was obtained. Thus, guanine may form soluble oligomers in the higher solubility state, although no evidence for oligomer formation exists. The relative solubility of the nucleobases against

adenine was calculated as shown in Fig. 1. Cytosine exhibited 8-fold higher solubility than adenine. Thymine and uracil exhibited about 3-fold higher solubility than adenine.

Solubilization effect of arginine on nucleobases

The solubility of nucleobases at pH 7.0 in the presence of arginine was measured as a function of arginine concentration (Fig. 2). Arginine increased the solubility of all the nucleobases concentration-dependently. The solubility in guanidine at pH 7.0 is also presented in Fig. 2. To our surprise, the solubilizing effects of guanidine on adenine were substantially weaker than that of arginine; although, for other nucleobases, the effect of guanidine and arginine were similar. It is therefore evident that arginine is stronger than or at least equivalent to guanidine in enhancing the solubility of nucleobases. These results point out not only the importance of the guanidinium group, shared by these two additives, in increasing the solubility of the nucleobases, but also the contribution of other parts that cause a difference between arginine and guanidine in modulating between the solubility of the nucleobases. Figure 2 also presents solubility data in glycine and NaCl solutions at pH 7.0. Addition of the additives above was either ineffective (i.e., for guanine, cytosine and uracil) or unfavorable (i.e., adenine and thymine), consistent with the weak salting-out property of these additives [21].

The solubilizing effects of 1 M arginine solution at pH 7.0 were compared for

different nucleobases. Figure 3 shows that 1 M arginine greatly increased the solubility of adenine: by about 2.5-fold. The effects of 1 M arginine on the guanine and cytosine solubility were moderate—about 2-fold, whereas those on thymine and uracil were smaller. The magnitude of the solubility enhancement of arginine was unrelated to the solubility of nucleobases in buffer solution, as represented by the result that cytosine and guanine were similarly affected by arginine despite their different solubilities in buffer solution (Figs. 1 and 3).

pH dependence of nucleobase solubilities in the presence of arginine

The measurements described above were done at pH 7.0, at which these nucleobases are electrically neutral. Adenine, guanine, and cytosine have respective pK_a values of 4.2, 3.3 and 4.6. They are positively charged in acidic solution. Figure 4 shows the pH dependence of the solubility of these nucleobases in citrate–phosphate buffer solution. Because the solubility measurement of these nucleobases could not be done exactly at the target pH because of pH changes after dissolution of the nucleobases, the pH shown in Fig. 4 is the final pH of the supernatant after dissolution. As expected, the solubility of electrically neutral thymine and uracil showed no pH dependence whether 1 M arginine was absent or present. The solubility of these two nucleobases was consistently higher in the presence of 1 M arginine. Arginine changes the net charge at the carboxyl pK_a, i.e., a

monovalent cation above ca. pH 2 and a divalent below this pH. The observed lack of pH dependence of thymine and uracil solubilities in 1 M arginine suggests that monovalent and divalent arginine have identical solubilizing effects on these nucleobases.

On the other hand, the solubilities of adenine, guanine, and cytosine exhibited a distinct pH dependence: i.e., the solubility of these nucleobases increased concomitantly with decreasing pH. Such increased solubility at lower pH is consistent with the increased electrostatic potential by the protonation of nucleobases, which is expected to make the nucleobases in solution phase more energetically stable. The solubility of adenine showed a peculiar pH dependence, with a sudden decrease at pH 3.3 followed by a gradual increase below pH 3.1. That solubility might be attributable to a phase transition of the precipitate: i.e., the chemical potential of the precipitate became lower at pH 3.3 for unknown reasons, leading to decreased solubility. Unexpectedly, the solubility of these nucleobases was lower in 1 M arginine than in buffer when compared at an identical pH in the acidic region, indicating that 1 M arginine actually destabilizes the protonated form of the nucleobases. The ratio of the solubility in 1 M arginine to that in buffer is shown for guanine and cytosine in Fig. 5. Because no data exist at identical pH values in the presence and absence of arginine for each nucleobase, as shown in Fig. 4, the solubility ratio was calculated by interpolation of the data points. The solubilizing effect of 1 M

arginine steeply decreased as the pH is lowered below ca. pH 4 for guanine and ca. pH 5 for cytosine. The solubilizing effect of 1 M arginine on guanine was lost at ca. pH 2.3, below which arginine decreased the solubility (i.e. solubility ratio less than 1). It is noteworthy that the midpoint of the ratio (ca. pH 3) was close to the pK_a of guanine (pK_a = 3.3). A similar trend was apparent for cytosine (pK_a = 4.6): the solubilizing effect was lost at pH 3.7 and the midpoint of the ratio was around pH 4. The observed different midpoint between these nucleobases and the fact that both monovalent and divalent forms of arginine had identical solubilizing effects on thymine and uracil suggest that the observed loss of solubilizing effects of arginine on guanine and cytosine at acidic regions are not caused by protonation of arginine, but by protonation of the nucleobases. Although not shown in Fig. 5, the observed suppression of adenine solubility by 1 M arginine below pH 4 is also expected to be responsible for the protonation (Fig. 4). The unfavorable interaction between arginine and protonated nucleobases might be attributed to their mutual electrostatic repulsion.

Solubility in different amino acid solutions

Solubilizing effects of 1 M arginine were compared with those of other amino acids at pH 7.0. Figure 6A shows the solubility in grams per 100 g solvent of nucleobases in 1 M amino acid solutions; the solubility unit differs from the molar expression used in Fig. 2.

Only arginine significantly increased the solubility for all five nucleobases. Glycine did not affect the solubility, irrespective of the nature of the nucleobases. Both aspartic acid and glutamic acid slightly decreased the solubility of these nucleobases. The effects of lysine were variable, resulting in no change for adenine, a slight increase for cytosine and guanine, and slight decreases for thymine and uracil. The effects of aspartic and glutamic acids are consistent with their preferential hydration on proteins [1,22].

Thermodynamic effects of amino acid solution can be compared better using transfer free energy, i.e., Gibbs free energy required to transfer the compound from buffer solution to the solution containing an additive. The weight-based solubility, such as that shown in Fig. 6A, was used to calculate the transfer free energy. Figure 6B presents the transfer free energy of the nucleobases from the buffer solution to 1 M amino acid solutions at pH 7.0. Here, comparison of transfer free energy of guanine in the absence and presence of the additives assumes that guanine retains the higher solubility state, as mentioned before, even in the presence of the additives. It is evident that arginine greatly decreased the transfer free energy of all nucleobases, indicating that arginine favorably interacts with these nucleobases. However, a possibility cannot be excluded that the addition of arginine altered the interaction of buffer components (50 mM citrate–phosphate) with the nucleobases, resulting in increased solubility. This possibility may be less likely, since

other amino acids showed no increased solubility. The stabilizing effect of arginine was in the order of adenine > guanine ≈ cytosine > thymine ≈ uracil. This order is related to the number of double bonds in the six-membered ring: i.e., a greater number of double bonds in the ring imply a more favorable interaction between arginine and the nucleobase. Glycine was neutral to these nucleobases because the transfer free energy was more or less zero. The transfer free energy was slightly positive for aspartic and glutamic acid, in particular against adenine and thymine. Lysine showed small favorable interactions only with guanine and cytosine.

DNA degradation

The effects of arginine on DNA digestion by DNase II were examined at 37 °C in 0.16 M acetate buffer (pH 5.0) as a preliminary experiment. A trend that the addition of 0.2 M arginine, but not NaCl, significantly inhibited DNA degradation was observed. Although this may be due to inhibitory effect of arginine on DNase II enzyme activity, another possibility could be the favorable interaction between arginine and nucleobases, which reduces affinity of the enzyme for the bases on DNA molecules.

Discussion

This paper described that arginine interacts favorably with nucleobases. Among the

amino acids tested, only arginine exhibited significant solubilizing and stabilizing effects on the nucleobases (Fig. 6), indicating that the guanidinium group of arginine is mainly responsible for the effect. Figure 6B shows that the transfer free energy of nucleobases decreases in 1 M arginine solution, i.e., the nucleobases are more stable in the arginine solution. The observed effects of arginine on nucleobases indicate that arginine increases the solubility not only of aromatic hydrocarbon-containing compounds but also of heteroaromatic compounds. The observed stronger effects on nucleobases with more double bonds imply that the favorable interaction of arginine with aromatic compounds arises from delocalized electron density. Although the physical mechanism of this interaction between arginine and electron cloud is not clear, the importance of such interaction in the observed arginine-enhanced solubility of nucleobases is evident from the pH dependence of the solubility (Fig. 4). Arginine decreased the solubility of adenine, guanine, and cytosine at acidic pH where they were protonated. Electrostatic repulsion between the positively charged arginine and the nucleobases should be responsible for the reduction of the favorable interaction as observed at neutral pH. It is likely that arginine is excluded from the nucleobases by the unfavorable electrostatic interaction and that such exclusion effects engender the salting-out effect of arginine on the nucleobases. Similar pH dependence has been observed with a salting-in salt, $MgCl_2$ [23]. This salt showed

favorable interaction with proteins at neutral pH, accompanied by salting-in of the proteins. The same proteins showed decreased solubility in the presence of $MgCl_2$ at low pH, where the proteins acquire positive charges. Concomitantly, $MgCl_2$ showed exclusion from the proteins at acidic pH.

Arginine has been used primarily in protein applications. The effects of arginine on proteins have been explained at least in part from its effects on aromatic hydrocarbon moiety, i.e., tryptophan, tyrosine and phenylalanine [6,24,25]. Other aromatic hydrocarbons, often found in drug substances, also show favorable interaction with arginine based on the interaction mechanism described above [17,18]. In this report, we described that arginine also increases the solubility of heteroaromatics, which also constitute many drug substances. Consequently, arginine might have wider applications as a solubility-enhancing additive.

The observed solubilizing effects of nucleobases suggest that arginine might affect the solubility or stability of nucleic acids composed of four nucleobases. In general, short nucleic acids undergo reversible cooperative thermal melting, leading to exposure of nucleobases to the solvent (Fig. 7). Favorable interaction of arginine with the nucleobases should stabilize the melted state, i.e. a single strand with the nucleobases more solvent-exposed. However, arginine, being cationic, should more favorably interact with

the backbone of the nucleic acids, which has the higher negative charge density. The overall effect of arginine on the reversible melting of oligonucleotides will therefore be determined as a fine balance between electrostatic (stabilizing single and double strand) and aromatic (stabilizing single strand) interactions with arginine. We previously observed that arginine enhances the stability of DNA at less than 0.2 M and destabilizes the DNA at higher concentrations [26]. The stabilization effect is attributable to the contribution of electrostatic interaction between arginine and double-strand DNA. The destabilization at higher concentration is caused by the increasing contribution of the favorable interaction between nucleobases and arginine. Polymerase chain reaction (PCR) technology involves the melting of nucleic acids and therefore exposure of the nucleobases to solvents. The observed favorable interaction of arginine with the nucleobases implies that arginine may have a marked influence on PCR processes.

We showed, although preliminary, that arginine inhibits DNA digestion by DNase II. The inhibition might be caused by decreased affinity of the enzyme for the nucleobases of the target DNA in the presence of arginine. The nucleobases had a lower free energy in the presence of arginine. Therefore, assuming that the nucleobases in the DNA, but not in the enzyme–DNA complex, interact with arginine, the binding constant of the enzyme to DNA is decreased by arginine. Perhaps for this reason, arginine caused slower kinetics of

DNA digestion.

We reported previously that arginine effectively elutes adenosine triphosphate (ATP)-dependent enzymes from ATP-affinity columns: the bound enzymes are normally eluted by free ATP [27]. This observation is explainable at least in part in terms of the favorable interactions between nucleobases and arginine. Crystal structures of nucleotide–enzyme complexes, e.g. nucleoside diphosphate kinase–adenosine diphosphate (ADP) complexes, showed that adenine moiety is involved extensively in the ADP binding to the enzyme [28]. Assuming that ATP is solvent-exposed in the column, but not in the ATP–enzyme complex, the observed elution of the enzyme can be accounted for in part by the decreased free energy of nucleobases in the presence of arginine.

Acknowledgments

The authors thank Dr. D. Ejima and Dr. S. Kuroda for valuable discussions. This work was partly supported by a Grant-in-Aid for Scientific research No. 18750140 from MEXT of Japan and the Tsukuba Industrial Liaison and Cooperative Research Center.

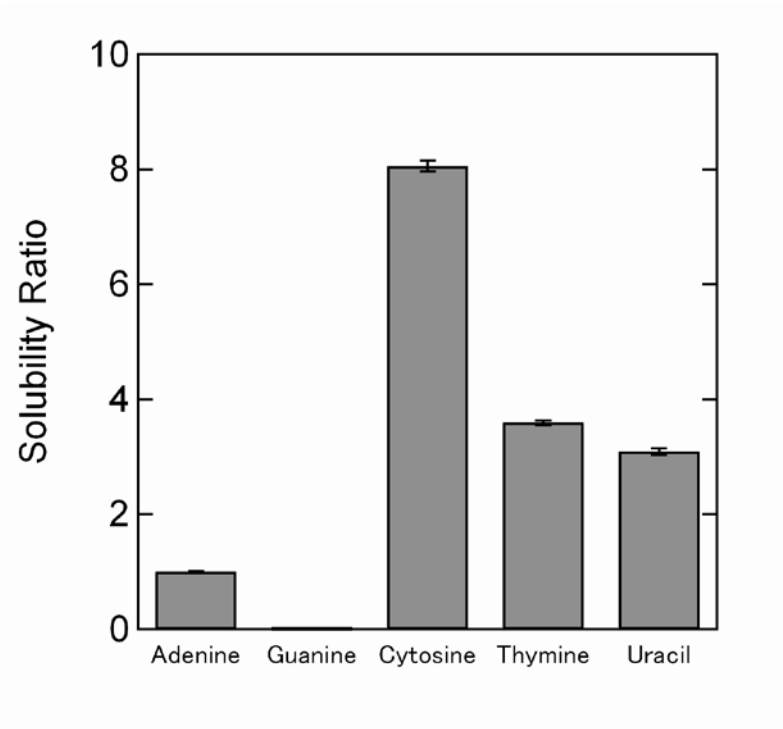

Fig 1. Ratios of nucleobase solubility to adenine solubility in buffer solution.

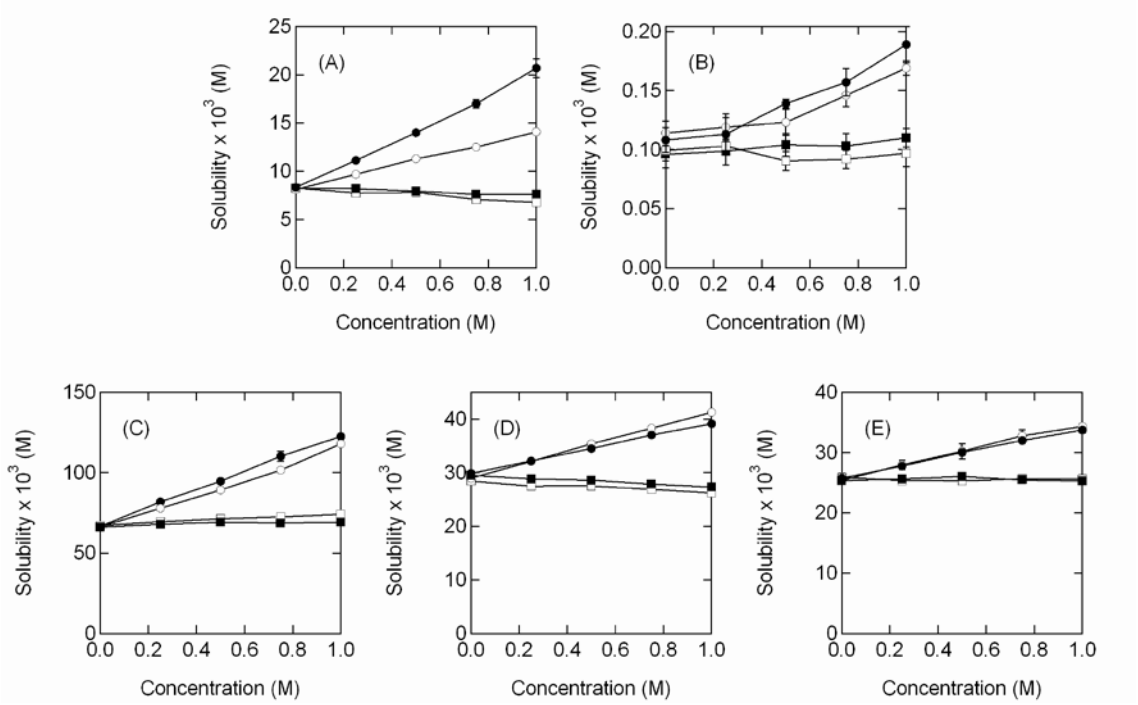

Fig 2. Solubilities of nucleobases in the presence of additives: (A) adenine; (B) guanine; (C) cytosine; (D) thymine; (E) uracil. Closed circles, arginine; open circles, guanidine; closed squares, glycine; open squares, NaCl.

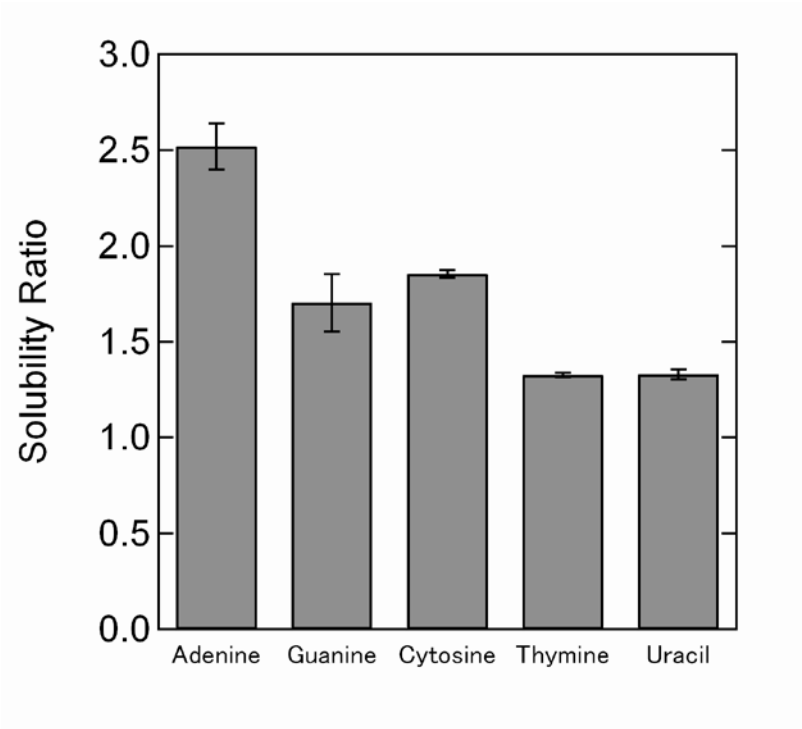

Fig 3. Ratios of nucleobase solubility in 1 M arginine solution to that in buffer solution.

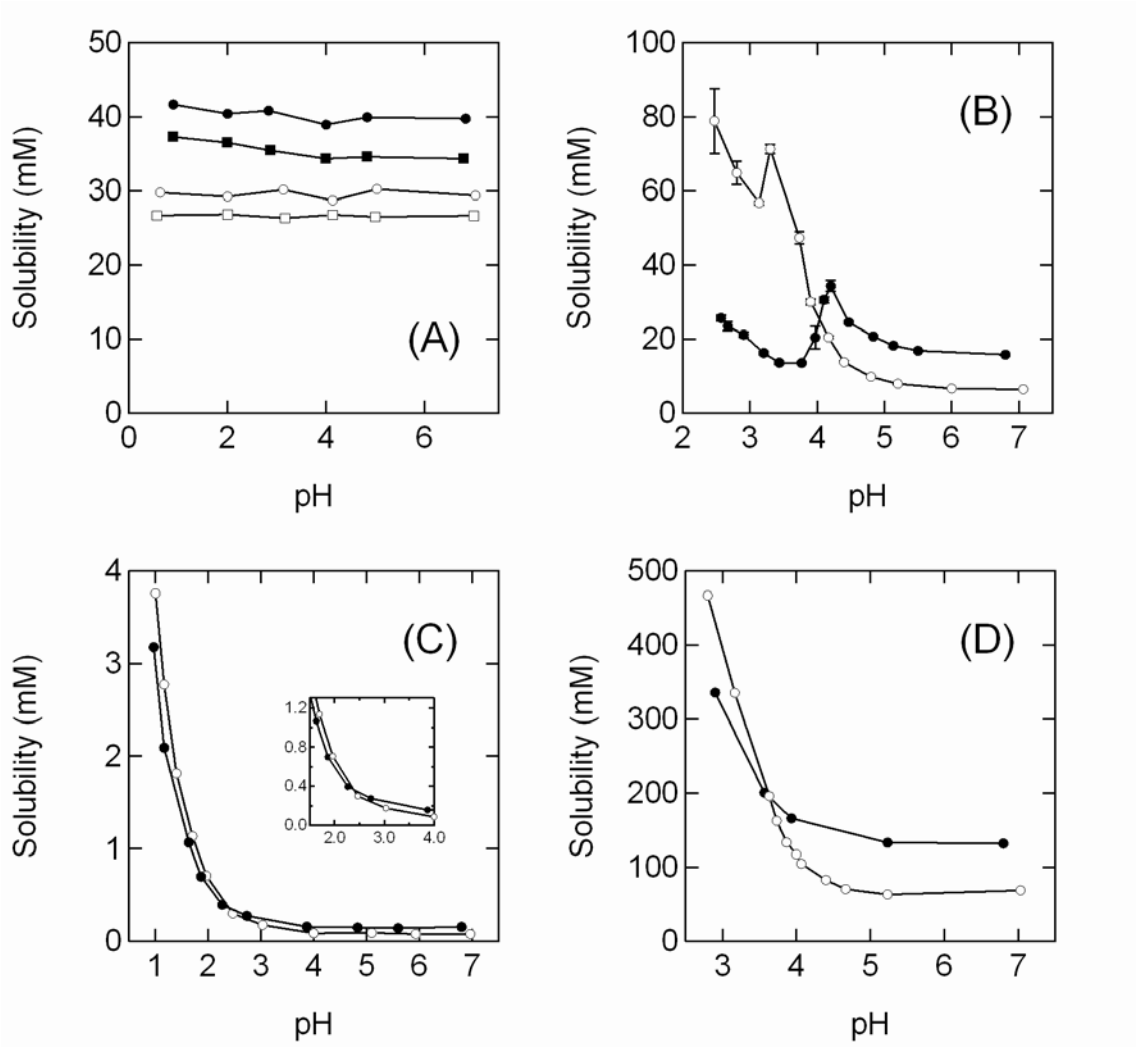

Fig 4. pH Dependence of the nucleobase solubilities in the presence (closed symbols) and absence (open symbols) of 1 M arginine: (A) circles, thymine; squares, uracil; (B) adenine; (C) guanine; (D) cytosine.

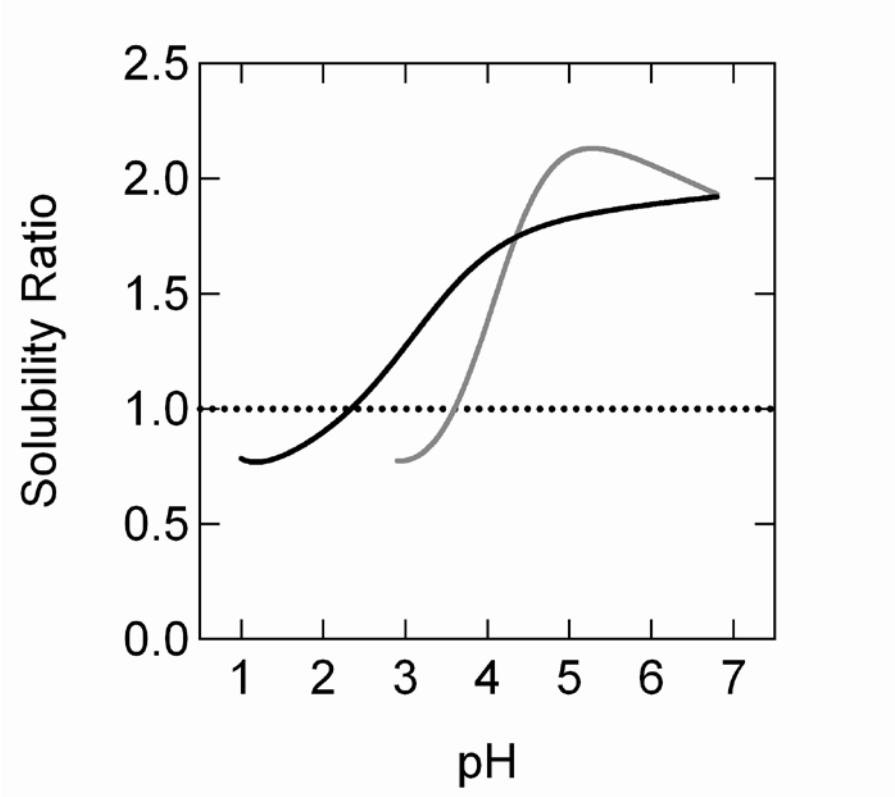

Fig 5. Solubility ratios of nucleobases in the presence of 1 M arginine to that in the buffer solution at identical pH. Black line, guanine; gray line, cytosine. Data are taken from Fig. 4 and calculated from interpolated values.

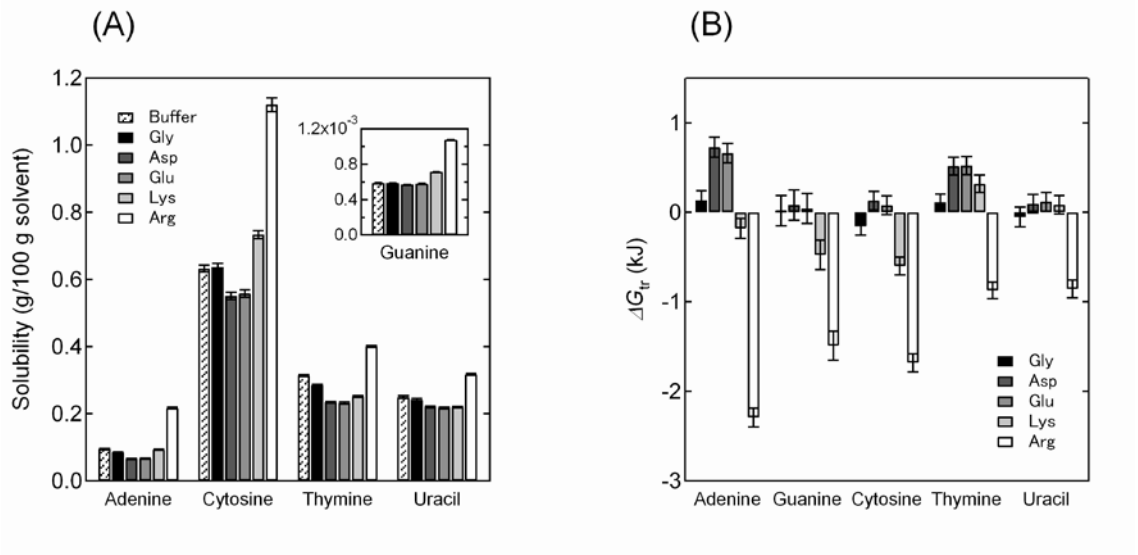

Fig 6. (A) Solubilities of nucleobases in 1 M amino acid solutions. (B) Transfer free energy of nucleobases from buffer solution to 1 M amino acid solutions.

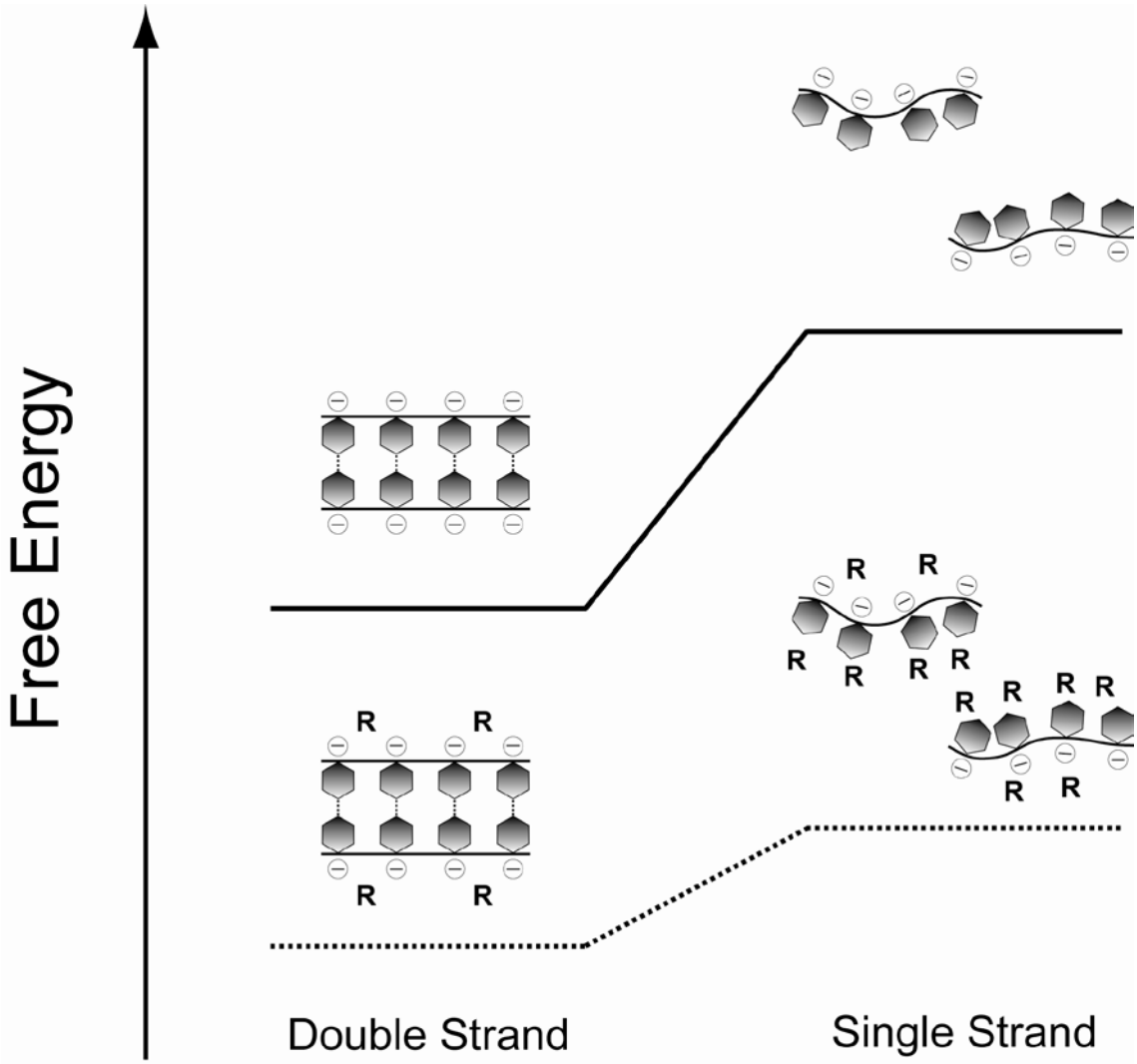

Fig 7. Free energy diagram of single-strand and double-strand DNA in the presence (dotted line) and absence (solid line) of arginine. Hexagons and R respectively depict nucleobases composing DNA backbone and arginine.

Table 1 Solubilities of nucleobases at pH 7.

Nucleobase	Solubility $\times 10^3$ (M)	Solubility^{ref} $\times 10^3$ (M) [a]
Adenine	8.20 ± 0.07	8.7 ± 0.1
Guanine	0.111 ± 0.004	0.039 ± 0.001
Cytosine	66.1 ± 0.5	65.8 ± 0.5
Thymine	29.4 ± 0.2	27.8 ± 0.6
Uracil	25.3 ± 0.4	23.8 ± 0.2

[a] Data from Ref. [19] for adenine and guanine and Ref. [20] for cytosine, thymine and uracil.

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